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<th>The prevalence and characterization of verotoxin-producing Escherichia coli isolated from cattle and pigs in an abattoir in Hong Kong</th>
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<td><strong>Author(s)</strong></td>
<td>Leung, PHM; Yam, WC; Ng, WWS; Peiris, JSM</td>
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<tr>
<td><strong>Citation</strong></td>
<td>Epidemiology And Infection, 2001, v. 126 n. 2, p. 173-179</td>
</tr>
<tr>
<td><strong>Issued Date</strong></td>
<td>2001</td>
</tr>
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</tbody>
</table>
The prevalence and characterization of verotoxin-producing *Escherichia coli* isolated from cattle and pigs in an abattoir in Hong Kong

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(Accepted 5 December 2000)

**SUMMARY**

The aim of the study was to define the prevalence of verotoxin-producing *Escherichia coli* (VTEC) in cattle and pigs in a Hong Kong abattoir. Faecal and carcass samples collected from 986 cattle and 487 pigs from an abattoir were tested for verotoxin (VT) by PCR and cytotoxicity assays. VTEC was isolated from 41.5 and 1.8% of cattle faecal and carcass samples and from 2.1 and 0.2% of porcine faecal and carcass samples, respectively. Amongst 409 VTEC isolates from cattle, 9 were serotype O157:H7 and *eaeA*+. The most prevalent *vt* genotype among bovine VTEC was *vt1 vt2*+ (73.8%) and in porcine VTEC was *vt2e*+ (30%). None of the porcine VTEC isolates and 9.3% of the bovine VTEC isolates was *eaeA*+. The non-O157 serogroup VTEC isolates carrying *eaeA* and *EHEC-hlyA* belonged to serogroups O172, O15, O84, O91, O110 and O121. The local dietary preference for pork or chicken (rather than beef), the low VTEC carriage in pigs, the rarity of additional virulence factors (*eaeA*) in VTEC isolated from cattle may explain the apparently low incidence of human diarrhoeal disease associated with VTEC in Hong Kong hitherto. However, the presence of non-O157 VTEC strains carrying the *eaeA* virulence marker in cattle highlights the fact that sole reliance on sorbitol-MacConkey agar for screening human VTEC isolates may underestimate the human disease burden. The changing dietary habits of the population in Hong Kong reinforce the need for continued vigilance.

**INTRODUCTION**

Verocytotoxin (VT) is a potent cytotoxin that inhibits eukaryotic protein synthesis and is a well-established virulence factor for *Escherichia coli* (VTEC) associated with human disease [1]. VTEC are geographically widespread and have been found to be associated with human disease ranging from bloody diarrhoea to the haemolytic-uraemic syndrome (HUS) [1]. The pathogenicity of VTEC is associated with additional virulence factors, the best known being the attaching-and-effacing (*eae*) A gene that encodes intimin, a 94 kDa outer-membrane protein [2]. Intimin enables VTEC to cause attaching-and-effacing lesions in the microvilli of the host intestine. Another virulence factor, the enterohaemorrhagic *E. coli* haemolysin A (*EHEC-hlyA*) gene, is encoded on a 60 MDa plasmid and is associated with EHEC strains [2]. Although Sandhu and colleagues reported that *EHEC-hlyA* was present in almost all *eaeA*-positive VTEC isolates obtained from animals [3], its role in the pathogenicity of VTEC is poorly understood.

VTEC is a naturally occurring organism in the gut flora of cattle [4] and has been isolated from the gut of other animals such as sheep, goats and pigs [1].
Outbreaks are associated with consumption of contaminated undercooked ground beef and vegetables or unpasteurized milk [2]. Serotype O157:H7, the best-known serotype of VTEC associated with human disease, was responsible for the foodborne outbreak that occurred in Japan in 1996 in which a total of 9578 cases were reported [5]. DNA patterns of the isolates from infected cases suggested a heterogeneous source of contamination.

The epidemiology of VTEC in man and animals has been studied in Western countries and Japan [6–8] but data from South East Asia and China are sparse. In this study, faecal and carcass samples were collected from cattle and pigs in an abattoir in Hong Kong, in an attempt to study the frequency of VTEC carriage in healthy livestock in the province. Local abattoirs process over 2000000 pigs and 50000 cattle annually [9]. Since the local community consumes more pork than beef, the carriage of VTEC in pigs was of particular interest. Other virulence factors associated with VTEC strains isolated were also examined.

MATERIALS AND METHODS

Collection of faecal and carcass samples

Rectal and carcass swabs were collected immediately after the slaughter and evisceration of animals in a Government abattoir in Hong Kong. The animals sampled were 986 adult cattle and 487 adult pigs transported to Hong Kong immediately prior to slaughter from various provinces in mainland China. The samples were collected monthly between August 1996 and December 1998.

Culture for VTEC

Rectal swabs were inoculated onto sorbitol–MacConkey (SMAC) agar (Oxoid Ltd, Basingstoke, UK) and incubated at 37 °C overnight. Swabs from carcasses were inoculated into VTEC enrichment broth (2 g tryptone, 1-12 g bile salt, 5 g lactose, 4 g K₂HPO₄, 1.5 g KH₂PO₄, 5 g NaCl and 0.02 g novobiocin per litre) and incubated overnight with gentle agitation at 37 °C. A loopful of each broth culture was heated to 94 °C overnight, with subsequent heating of the broth culture at 94 °C for 1 min and 3 min at 43 °C and 1 min at 72 °C respectively, followed by a final extension period of 7 min at 72 °C. Amplified products were visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel. PCR-amplified DNA was transferred from the agarose gel to Hybond-N nylon membranes (Amersham, Arlington Heights, IL, USA) by Southern blotting [11]. The membranes were hybridized at 45 °C overnight, with probes 428-I, 428-II and vt2e (Table 1) for detection of vtI, vt2 and vt2e (GenBank accession number M29153 [12]) respectively. These probes were labelled using the DIG DNA 3’ end-labeling and detection kit (Boehringer–Mannheim, Mannheim, Germany). The overnight hybridization was followed by two stringency washes of the membrane at 50°C in 0.2 X SSC (0.5 M NaCl, 3 x SDS trisodium citrate, pH 7.0) with 0.1% SDS for 15 min. Labelled DNA was visualized by anti-digoxigenin enzyme-linked immunosorbent assay.

Detection of VT production by vero cell assay

Samples positive for vt by PCR were confirmed by a vero-cell cytotoxicity assay [13]. For each specimen, 10 colonies were picked from the SMAC plate and cultured in non-selective nutrient broth. One ml of each broth culture was centrifuged at 13000 r.p.m. for 15 min; 50 µl of supernatant was diluted 1 in 5 in Eagle’s minimal essential medium and added to vero cell monolayers grown in 96-well microtitre plates. The plates were incubated at 37 °C overnight in 5% CO₂ and cells were examined for cytopathic effect (CPE) using an inverted microscope. Supernatants producing CPE of ≥ 50% of the monolayer were considered VT producers.
Table 1. Primers and probes used for detection of virulence genes of VTEC isolates

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Sequence (5′–3′)</th>
<th>Location (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK1</td>
<td>vt</td>
<td>TTTACGATAGACTTCTCGAC</td>
<td>311–330</td>
<td>10</td>
</tr>
<tr>
<td>MK2</td>
<td>vt</td>
<td>CACATATAAATTATTTTCGTC</td>
<td>515–535</td>
<td></td>
</tr>
<tr>
<td>hlyAF</td>
<td>EHEC-hlyA</td>
<td>GCATCATCAAGCGTACGTTCC</td>
<td>70–90</td>
<td>14</td>
</tr>
<tr>
<td>hlyAR</td>
<td>EHEC-hlyA</td>
<td>AATGAGCCAAGCTGGTTAAGCT</td>
<td>582–603</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>eaeA</td>
<td>TCGTCACAGTTGCAGGCCTGGT</td>
<td>803–824</td>
<td>15</td>
</tr>
<tr>
<td>C2</td>
<td>eaeA</td>
<td>CGAAGTCTTATCCGCCGTAAAGT</td>
<td>1890–1912</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>428-I</td>
<td>GATAGTGGCTCAGGGGATAA</td>
<td>428–441</td>
<td>10</td>
</tr>
<tr>
<td>Probe</td>
<td>428-II</td>
<td>AACCACACCCACGGCAGTTA</td>
<td>428–447</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>vt2e</td>
<td>AATCATACACCACCAGGAAG</td>
<td>431–450</td>
<td>This study</td>
</tr>
</tbody>
</table>

Detection of eaeA and EHEC-hlyA genes in VTEC isolates

VTEC isolates confirmed by vero cell assay were studied for their eaeA and EHEC-hlyA genes by multiplex PCR. The primers were based on the central conserved region and the 5′ region of the respective genes [14, 15]. The PCR reaction mix was identical to that for vt gene detection. Amplification conditions were as follows: 94 °C for 12 min followed by 30 cycles of amplification for 1 min each at 94 °C, 55 °C and 72 °C. Primers and probes for detection and identification of virulence genes in VTEC are listed in Table 1. eaeA-positive strains were further confirmed by fluorescence actin stain (FAS), as described previously [13].

Serotyping of isolates

All VTEC isolates identified by vero cell assay were confirmed by traditional biochemical tests. All sorbitol non-fermenting strains were serotyped using the E. coli O157:H7 Latex Test kit (Remel, Lenexa, KS, USA). Non-O157 VTEC isolates harbouring eaeA and EHEC-hlyA genes were serotyped at the Microbiological Diagnostic Unit, Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia.

Pulsed-field gel electrophoresis of O157:H7 isolates

The method of Cameron and colleagues [16] was used with minor modifications in the preparation of DNA plugs. A small portion of the plug (2 × 7 mm) was digested with 16 units of restriction enzyme XbaI (Amersham Pharmacia Biotech, Uppsala, Sweden) at 37 °C overnight. Electrophoresis was performed in 1% agarose in 0.5× TBE (12 °C buffer temperature) at 6 V/cm in a CHEF MAPPER system (Bio–Rad, Hercules, California, USA) with pulse times increasing from 5–50 s over a period of 20 h.

RESULTS

Prevalence and characteristics of VTEC in abattoir animals

VTEC were isolated from 409 (41.5%) faecal and 18 (1.8%) carcass samples from 986 cattle, and from 10 (2.1%) faecal and 1 (0.2%) carcass samples from 487 pigs (Table 2). Only four (0.41%) cattle yielded VTEC from both faeces and carcasses. PCR revealed a higher VTEC carriage in both species, particularly in carcasses (Table 2). The carriage rate in cattle appeared to be lower between May to August (data not shown) and highest in February and November.

Nine isolates of O157:H7 were isolated from cattle, one from a carcass and the remainder from faeces (Table 3). The O157:H7 isolate from the cattle carcass did not produce VT. All nine O157:H7 VTEC strains were sorbitol non-fermenting while 81% (324/401) of the non-O157 VTEC fermented sorbitol. In addition, one third of the non-O157 VTEC carrying principal virulence factors also fermented sorbitol. VTEC strains with eaeA were positive in the FAS test. Serotypes of all 30 non-O157 VTEC isolates carrying the principal virulence factors (vt, eaeA and EHEC-hlyA genes) isolated from cattle were determined and they were O172:H-, O15:H-, O91:H14, O84:H-, O110:H16, O121:H19, O rough:H-, O rough:H31 and O non-typeable:H- (Table 3).

Genotypic profile of the VTEC isolates

The genotypes of the VTEC isolates from cattle are summarized in Table 3. The most frequently isolated
Table 2. Carriage rate of VTEC in cattle and pigs detected by PCR and vero cell assay

<table>
<thead>
<tr>
<th></th>
<th>Cattle</th>
<th></th>
<th>Pigs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Faeces</td>
<td>Carcass</td>
<td>Faeces</td>
<td>Carcass</td>
</tr>
<tr>
<td></td>
<td>(No. = 986)*</td>
<td>(No. = 986)</td>
<td>(No. = 487)</td>
<td>(No. = 487)</td>
</tr>
<tr>
<td>PCR</td>
<td>62.5%</td>
<td>11.4%</td>
<td>5.1%</td>
<td>2.3%</td>
</tr>
<tr>
<td>Vero cell assay†</td>
<td>41.5%</td>
<td>1.8%</td>
<td>2.1%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

* No., number of samples.
† The percent values for vero cell assay are also the total numbers of VTEC isolates.

Table 3. Genotypic profile of VTEC strains isolated from cattle

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>Number of strains</th>
<th>vt-1</th>
<th>vt-2</th>
<th>eaeA</th>
<th>EHEC-hlyA</th>
<th>Verotoxicity</th>
</tr>
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<tbody>
<tr>
<td>O157:H7</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O172:H-</td>
<td>10</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>O121:H19</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O111:H16</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O91:H14</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O84:H-</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O15:H-</td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O15:H-</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O rough:H-</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O rough:H31</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>O non-typable:H-</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other non-O157:H7</td>
<td>112</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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Table 4. Genotypic profiles of VTEC isolated from rectal swabs from pigs

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>vt1</th>
<th>vt2</th>
<th>vt2e</th>
<th>eaeA</th>
<th>EHEC-hlyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

bovine *vt* genotype was *vt1*”*vt2*” (73.8%). Only one strain (0.24%) carried *vt1*” alone. One verotoxic isolate did not hybridize with the *vt1*-, *vt2*-, or *vt2e*specific probes. This isolate was also negative in PCR for *vt2c* (results not shown). The virulence markers *eaeA*” and *EHEC-hlyA*” were present in 9.3% and 43.3% respectively, of the bovine VTEC strains. Thirty non-O157 and eight O157:H7 strains harboured *vt*, *eaeA* and *EHEC-hlyA* genes. One O157:H7 isolate was non-verotoxic but carried both *eaeA* and *EHEC-hlyA* genes.

The genotypic profiles of VTEC isolated from pigs are summarized in Table 4. None of the porcine VTEC strains carried the *eaeA* gene and three strains carried *vt2e* gene alone. The most prevalent genotype was *vt2e*”*eaeA*” *EHEC-hlyA*”, three isolates collected during the same abattoir visit belonged to this genotype. The next most common genotype was *vt1*”*eaeA*” *EHEC-hlyA*”. Two isolates fell into this category and they were isolated during different abattoir visits (Table 4).

Pulsed-field gel electrophoresis of O157:H7 isolates

Seven PFGE patterns were identified from the nine O157:H7 isolates and were designated A–G (Fig. 1). Isolates recovered from different abattoir visits had
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Fig. 1. Pulsed-field gel electrophoresis of O157:H7 isolates. Lanes 1 and 13, phage lambda DNA ladder size marker (Bio–Rad). Lane 2, O157:H7 (WHO strain). Lane 3, *E. coli* ATCC25922 (non-O157:H7 control). Lane 4, pattern A. Lanes 5 and 6, pattern B. Lanes 7 and 9, pattern C. Lane 8, pattern D. Lane 10, pattern E. Lane 11, pattern F. Lane 12, pattern G.

Table 5. PFGE patterns and vt genotypes of O157:H7 strains isolated from cattle at various abattoir visits

<table>
<thead>
<tr>
<th>Date of abattoir visit</th>
<th>Number of samples</th>
<th>PFGE pattern</th>
<th>vt genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>August 1996</td>
<td>1</td>
<td>A</td>
<td>No vt gene detected</td>
</tr>
<tr>
<td>April 1998</td>
<td>2</td>
<td>B</td>
<td>vt1 vt2*</td>
</tr>
<tr>
<td>November 1997</td>
<td>2</td>
<td>C</td>
<td>vt2*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>D</td>
<td>vt1 vt2*</td>
</tr>
<tr>
<td>August 1998</td>
<td>1</td>
<td>E</td>
<td>vt1 vt2*</td>
</tr>
<tr>
<td>January 1997</td>
<td>1</td>
<td>F</td>
<td>vt2*</td>
</tr>
<tr>
<td>June 1998</td>
<td>1</td>
<td>G</td>
<td>vt2*</td>
</tr>
</tbody>
</table>

Table 5 shows different patterns (Table 5), two isolates from a visit in April 1998 belonged to pattern B. Three isolates from a visit in November 1997 showed two PFGE patterns, C and D. The latter isolate had a different vt genotype.

**DISCUSSION**

In the present study, 41.5% of cattle harboured VT3C as determined by vero cell assay. The large discrepancy between PCR and the vero cytotoxicity assay may be partly accounted by the novobiocin-containing enrichment medium used for carcass samples, as the medium may be inhibitory to some VT3C strains [17]. In the evisceration process, the carcass is sprayed with hot water. This may kill contaminant bacteria and may explain the discrepancy between PCR (which can detect non-viable VT3C) and culture in the carcass samples. For the purposes of this study, only those specimens that were vero-cytotoxic were considered further.

The high carriage rate of VT3C in cattle also indicated that they were reservoirs for transmission of the organism. In other geographic areas, VT3C carriage rates in healthy cattle have been reported to be 34% in France [18], 24.3% in Japan [7], 53% in Sri Lanka [19] and 9.5% in Canada [8]. Our highest carriage rate occurred during the coolest months, which contrasts with the finding in France where the highest VT3C prevalence was in summer [18].

O157:H7 is the best known VT3C serotype associated with human diseases [2] and its presence in healthy animals and meat products has been reported by others [18, 20]. Here, the O157:H7 serotype was detected in 0.9% of the faecal samples from cattle, a rate comparable to that reported by Pradel and colleagues [18]. Eight O157:H7 isolates possessed the principal virulence factors (vt, eaeA and EHEC-hlyA). One of the isolates was vt− but eaeA+ and EHEC-hlyA+. Loss of vt gene probably occurred during subcultivation [21]. Nevertheless, vt+ eaeA+ O157:H7 and O26:NM have been isolated from healthy and diarrhoeal animals respectively [4, 22] and the pathogenicity of such strains remains to be defined. The PFGE pattern of isolates collected during one abattoir visit (presumably from the same farm) was identical but there were diverse patterns of isolates from different abattoir visits (Fig. 1).

The predominant vt genotype has been shown to vary in different geographical locations [18, 19, 23, 24] but the most prevalent vt genotype encountered in our cattle was vt1 vt2*. In this study, one verotoxic strain did not belong to vt1, vt2, vt2e or vt2c and this isolate requires further study. The predominance of vt2 (either alone or together with vt1) in our VT3C is noteworthy as VT2-producing VT3C were highly associated with HUS [25]. In our previous study on 1003 faecal samples from diarrhoeal patients in Hong Kong, of five VT3C isolated, two O157:H7 and one O rough:H45 harboured vt2 [26]. In this study, the majority of bovine non-O157 VT3C isolates were negative for eaeA. Such eaeA− VT3C have been reported to cause HUS in humans [27, 28]. Human isolates in Hong Kong have included O rough:H45 and O157:NM eaeA− strains [26], indicating that eaeA− VT3C may harbour virulence factors outside
the locus of enterocyte effacement. Such potential virulence factors include the astA and katP, which encode for a heat-stable enterotoxin and a catalase-peroxidase respectively. These strains have been found in animals and asymptomatic human carriers [29].

Previous studies showed that the EHEC-hlyA genotype was more frequently isolated from haemorrhagic colitis (HC) and HUS patients, indicating that this gene might play a role in the disease process [2]. In 66 animal VTEC isolates, the EHEC-hlyA and vt genes were closely associated, serving as a useful epidemiological marker for rapid selection of VTEC [30]. However, only 43.3% of VTEC from our cattle carried the EHEC-hlyA gene.

Pigs were included in this study as pork is a major source of meat consumed by the local population. We found that both the VTEC carriage and carcass contamination rates in healthy pigs were low. Our finding agrees with that of other investigators [23, 24], suggesting that pigs are not an important reservoir of VTEC. The O157:H7 serotype was not isolated from these strains. The cooperation of the staff at the Government abattoir is also gratefully acknowledged. This work was supported by a grant from the Hong Kong Research Grants Council (HKU 7314/97M) and a SPACE Research Grant Award (21386308.03982.70300.420.01).

Non-O157 VTEC carrying the virulence factors eaeA and EHEC-hlyA were serotyped (Table 3). One-third of these strains belonged to serotype O172, which has been isolated from one patient with HC in America [32] but was infrequently encountered in both humans and animals elsewhere [33, 34]. The first reported O172 strains harboured vt2 as did the O172 isolates described here. O15 is a common serotype in rabbit diarrhoea E. coli [35]. However, O15 strains can also be encountered in EHEC and are associated with cattle infection [36]. Serotype O91 is known to cause HUS in humans [27] and this serotype has been reported in healthy and diseased animals [37]. The O84, O110 and O121 serotypes have been isolated in diarrhoeal calves and humans [37–39]. This group of VTEC isolates may be potential intestinal pathogens causing a human public health hazard in Hong Kong and they may go unrecognized in human clinical specimens if reliance is placed solely on sorbitol–MacConkey agar for screening VTEC isolates. This, together with the changing dietary habits of the population reinforces the need for continued vigilance for outbreaks of VTEC in the humans.

ACKNOWLEDGEMENTS

We thank Wong Ka-wing for his excellent assistance, as well as Martin Tsang and the staff of the School of Professional and Continual Education, University of Hong Kong. We also thank Dr Karl Bettelheim from the Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia for serotyping the VTEC strains and Dr N. A. Stockbinnie from the Centers for Disease Control and Prevention, Atlanta, GA, USA, for providing the standard VTEC strains. The cooperation of the staff at the Government abattoir is also gratefully acknowledged. This work was supported by a grant from the Hong Kong Research Grants Council (HKU 7314/97M) and a SPACE Research Grant Award (21386308.03982.70300.420.01).

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